ASSESSMENT OF MOSQUITOFISH (*GAMBUSIA AFFINIS*) DOWNSTREAM OF DOMESTIC WASTEWATER EFFLUENTS IN THE BAYOUS OF HARRIS COUNTY

A Thesis

by

CRYSTAL DANYLLE WATKINS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2011

Major Subject: Wildlife and Fisheries Sciences

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Approved by:

Co-Chairs of Committee,	Kirk O. Winemiller
	Miguel Mora
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ABSTRACT

Assessment of Mosquitofish (*Gambusia affinis*) Downstream of Domestic Wastewater Effluents in the Bayous of Harris County. (December 2011)

Crystal Danylle Watkins, B.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Kirk O. Winemiller
Dr. Miguel A. Mora

The introduction of pharmaceuticals and personal care products (PPCPs) to aquatic systems has impacted development and reproductive health of fish in many regions of the world. This study investigated western mosquitofish in the bayou systems of Harris County, Texas for evidence of morphological and reproductive abnormalities. Mosquitofish were sampled above and below WWTPs on five streams during May and August 2010, and specimens were dissected and analyzed for reproductive (egg/embryo weight, embryo/egg number and embryos staging), morphological (liver and gonad weight, body length, gonadosomatic index and hepatosomatic index) and histological indicator variables. In addition, water samples were analyzed for concentrations of PPCPs. Spatial and temporal variation was observed among all indicator variables, however no consistent differences were found above versus below WWTP discharges. Histopathology showed no evidence of lesions or presence of intersex individuals.

Chemical analysis revealed a variety of pharmaceuticals and anthropogenic chemicals present in the Houston area waterways, however all were at concentrations lower than those known to cause impacts to fishes. These results suggest that the current concentrations of chemicals being discharged from WWTPs into headwater reaches of streams in the suburban area of west Houston are below levels that impact the physiology of male and female mosquitofish.

DEDICATION

I dedicate this thesis to my family. Thanks for the moral support and for the lesson that life is to be taken with small steps at a time.

ACKNOWLEDGEMENTS

I would like to thank Dr. Kirk Winemiller, for his encouragement, his guidance and support throughout the production of this research and thesis. Thanks for always pushing me to do more than I could ever imagine. I also would like to thank my co-advisor, Dr. Miguel Mora, for his valuable insight into the field of ecotoxicology and for his help in my research as well as in extra-curricular activities. Thanks to Dr. Duncan MacKenzie for serving on my committee and for help and advice on my research. Thanks to Dr. Bryan Brooks and Bowen Du of Baylor University and Dr. David Phalen of The University of Sydney for help analyzing water samples and histological samples, respectively. Thanks to my past and present lab members for putting up with my millions of questions and for giving me advice on everything from research to coursework to life. I am grateful that I had the chance to know some of the best people I've ever known. Special thanks goes to Chevaun McCray, my partner-in-crime, for all those trips to Houston off-roading in your truck and for always willing to be up for an adventure, even in the presence of "Houston otters". Thanks to those who have helped me in the field: Carmen Montana, Elizabeth Carrera, Alejandra Maldonado, and Kaila Patton; I hoped I showed you that the bayous are not as bad as they seem. Thanks to my family for being my cheerleaders, my therapists, my editors, my sounding board, and my rock when I needed it the most. I gratefully acknowledge the International Sportfish

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NOMENCLATURE

WWTP	Wastewater Treatment Plant
PPCP	Pharmaceutical and Personal Care Products
SL	Standard Length

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1. INTRODUCTION

1.1 Background

Introduction of anthropogenic chemicals into aquatic and terrestrial ecosystems has been shown to have significant impacts on wildlife {Sumpter, 1995 #78}. The classic case study of environmental pollution was dichlorodiphenyltrichloroethane (DDT), an organochlorine pesticide that was used worldwide, particularly in the United States in the early to mid 1900s. DDT was found to have devastating effects on wildlife and potentially on humans through disruption of reproductive physiology {Beard, 2006 #75}. Rachel Carson's *Silent Spring* and subsequent research findings led to the banning of DDT in the United States, the United Kingdom, and other countries. Presently, at least 19 compounds have been targeted for restriction due to potential harmful effects on humans and the environment {Maurice, 2001 #60;Longnecker, 1997 #71}.

Aquatic species may be particularly vulnerable to chemical pollution. Although a large body of research has shown that chemicals are making their way into water bodies around the world {Howell, 1980 #23;Toft, 2003 #22}, their effects on wildlife still have not been documented for most regions of the United States. Surface-water pollution has contributed to habitat degradation, novel selection pressures, reproductive anomalies and

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This thesis follows the style of Ecology.

population decline among aquatic organisms {Kidd, 2007 #53}. Endocrine- disrupting compounds (EDCs) are particularly concerning because they can cause physiological disruption, such as reproductive abnormalities, in fish and other aquatic organisms {Nichols, 1999 #74}. Among EDCs associated with wastewater treatment plant (WWTP) effluents are pharmaceuticals and personal care products (PPCPs), chemicals that have been reported to directly impact aquatic organisms {Purdom, 1994 #35}. Given the many recent reports of intersex organisms {Angus, 2002 #167;Kidd, 2007 #53}, altered reproductive behavior {Kime, 1998 #59}, and population declines {Kidd, 2007 #53} of aquatic organisms in habitats receiving WWTP effluents, it seems clear that PPCPs are a problem that can no longer be ignored.

Treatment plants in large urban centers in the United States are presumed to release significant concentrations of PPCPs {Brooks, 2005 #97} that have the potential to cause negative effects on aquatic organisms. Algae can be highly sensitive, with PPCPs inhibiting growth and causing higher toxin concentrations levels when compared with other organisms tested {Harada, 2008 #124}. Amphibians are also especially vulnerable due to their thin, permeable skin that readily absorbs dissolved chemical compounds {Norris, 2011 #125}. Fraker and Smith {, 2004 #121} found that tadpoles exposed to biologically significant amounts of the antibacterial agent, triclosan, were less active than controls and also had a lower survival. Exposure to triclosan and caffeine influences the startle response on the tadpoles, with a lower response in triclosan and a higher response in caffeine. Triclosan exposure altered activity levels of frogs, which in turn led to higher predation rates and lower reproductive success {Fraker, 2004 #121}.

Among aquatic toxicity studies, fish have been used in a variety of ecotoxicology studies with a variety of responses to toxin exposure.

The release of PPCPs into aquatic systems has also been found to cause negative impacts in fish. One study found that eight out of ten targeted antidepressants measured in effluent-dominated streams were found in white sucker, *Catostomus commersonii,* brain tissue; the compounds presumably originated from bed sediment and/or environmental exposure via water {Schultz, 2010 #119}. Studies in the United Kingdom have pointed toward PPCPs as the cause for sex change in wild roach, *Rutilus rutilus* {Jobling, 1998 #120}. 17-alpha-ethinylestradiol (EE_2), a synthetic estrogen, discharged from wastewater treatment plants, was suggested to be the endocrine disrupting compound {Kime, 1999 #38;Kime, 1999 #38;Jobling, 2002 #129}. Lange et al. {, 2009 #32} found that wild roach exposed to EE_2 for two years were completely feminized. Fathead minnows, *Pimephales promelas,* are frequently used in toxicology studies with responses ranging from changes in gonad gene expression downstream of a WWTP {Garcia-Reyero, 2008 #126} to feminization of males expose to EE_2 {Kidd, 2007 #53}. Studies with mosquitofish have found a reduction of the male sex organ (gonopodium) size as well as a decrease in male sexual behavior {Doyle, 2002 #127}. Spironolactone, an anti-androgenic pharmaceutical, has been shown to masculinize female mosquitofish by the elongation of the anal fin into a gonopodium-like structure {Raut, 2011 #128}. One study found that large doses of EE_2 have the potential to cause the extirpation of local fish populations {Kidd, 2007 #53}. The variety of physiological responses to PPCP exposure makes fish a useful study organism in ecotoxicology studies.

1.2 Chemicals in Wastewater Treatment Plant Effluents

Chemicals found in surface waters originate from both point and non-point sources including agriculture, urban runoff and WWTPs. Recently, concern has focused on WWTPs as important point sources of environmentally-active synthetic chemicals such as PPCPs. A mixture of secondary and tertiary treatment is used in water treatment plants in the United States {Iken, 2011 #136}. Secondary treatment involves removing dissolved and suspended biosolids through the use of microorganisms. In tertiary treatment, the water is subsequently disinfected through the use of chemicals or the use of treatment wetlands {Iken, 2011 #136}. Advanced technologies, such as activated carbon and reverse osmosis, are potential options for removal of PPCPs {Snyder, 2003 #130}, but future research on the efficiency of these treatments is needed.

PPCPs have been detected in surface waters worldwide, and the continuous release from WWTPs in aquatic systems is a growing concern given the increasing use of prescription drugs throughout the world. Entry of these chemicals into sewage is primarily via human excretion. In a study of five effluent-dominated streams in the United States, Ramirez {, 2009 #99} found the presence of 5 pharmaceuticals, including anti-depressants and high blood pressure medicine, in fish fillets collected from these sites. It is known that the wastewater treatment process does not completely eliminate these compounds {Heberer, 2002 #135}, which makes trying to understand the processes through which they affect aquatic species all the more important.

1.3 Biomarkers of PPCPs Exposure in Fish

Biomarkers are a commonly accepted way to identify both acute and chronic exposure to PPCPs {Hutchinson, 2006 #165}. Biomarkers are defined as a distinguishable reaction to a chemical that can cause a physical, behavioral, or genetic change. Because one of the major modes of action of PPCPs is estrogenic {Snyder, 2003 #131}, one useful biomarker in fish is the induction of vitellogenin, an egg-yolk protein produced in the liver of females during oogenesis {Larsson, 1999 #27}. A common estrogenic compound found in effluent discharge is EE_2, the potent synthetic estrogen that is used in oral contraceptive pills {Ternes, 1999 #70}. Mature channel catfish exposed to estrogenic compounds at one WWTP effluent-dominated stream had a serum vitellogenin much higher than the reference values, with 220% in the fall sample and 480% in the spring sample {Tilton, 2002 #133}. The presence of intersex fish, individuals with both ovarian and testicular tissue, was a useful biomarker in Jobling's {, 1998 #120} study of roach in rivers receiving treated sewage effluents. When analyzing gonadal tissues from "male" roach in the study areas, it was found that they were actually intersex individuals with gonads of both sexes {Jobling, 2002 #129}. In areas exposed to EDCs, the intersex condition can reduce fish reproductive success {Jobling, 2002 #129;Kime, 1995 #41}.

Not only has PPCPs been found to cause physiological damage, but they it can also cause an alterations in behavior. Nassef {, 2010 #158} found that Japanese medaka fish (*Oryzias latipes*) exposed to the PPCPs had altered feeding behavior and swimming speed. The time to eating midge larvae (feeding behavior) was increased when exposed to diclofenac and carbamazepine. The anti-bacterial agent, triclosan, significantly decreased the mean swimming speed. Other studies {Orvos, 2002 #159} have confirmed a change in behavior when exposed to pharmaceuticals and have concluded that these behavioral indicators are important for evaluating the sublethal effect of these compounds {Nassef, 2010 #158}.

Physical, morphological, and histological biomarkers such as lesions and abnormalities of gonads and other body parts are also useful in studying toxicological effects of chemicals on fish. Angus {, 2005 #31} found that male western mosquitofish *(Gambusia affinis)* exposed to EE_2 had a reduced gonopodial length and the testes failed to grow and develop normally. Batty and Lim {, 1999 #16} found that males inhabiting streams receiving WWTP effluents in Australia had a shorter gonopodia. Because insemination can only occur with a fully developed gonopodium, this condition should result in lower reproductive success {Bisazza, 1996 #103}. Rawson {, 2008 #91} suggested that abnormal morphology of hemal spines at the tip of the gonopodium could be attributed to the estrogen content of sewage effluents. The study also found that there was a decrease in the proportion of males downstream of a WWTP compared to the control site {Rawson, 2008 #91}. Overall, it seems that the chemicals being discharged from WWTPs are causing morphological, reproductive, behavioral and other alterations that have broader impacts on fish, including reduction in fecundity {Nash, 2004 #160} and population crashes {Kidd, 2007 #53} on fish populations.

1.4 Study Area

My project examined fish collected from stream reaches exposed to domestic WWTP effluents in comparison to fish collected from upstream reaches that do not receive these effluents. Samples were collected from sites within tributaries of Buffalo Bayou, Greens Bayou, and Cypress Creek (Fig.1) within the Houston metropolitan area, Harris County, Texas. The study sites were chosen to evaluate the effect on fish of effluents from the uppermost WWTP on each stream. The Houston Bayou Stream System is located in the San Jacinto River Basin and spans 4023 kilometers. In the beginning of the 19th century, the bayous, especially Buffalo Bayou, were used for navigation to Galveston Bay (Harris County Flood Control District 2009). Pollution has always been an issue associated with Houston's bayous, starting in the early 1800s with concern over saw mill pollution {Smyer, 2008 #64}. With the population growth of Houston in the late 1800s, many residents started to complain about the condition of the bayous, and to this day it is still a major concern of residents {Turner, 2008 #89}. After passage of the Clean Water Act of 1972, the state of Texas sued the city of Houston for dumping untreated sewage into the bayous {Smyer, 2008 #64}. This led to a $3 billion sewage overhaul that has vastly improved the water quality of Buffalo Bayou {Smyer, 2008 #64} .

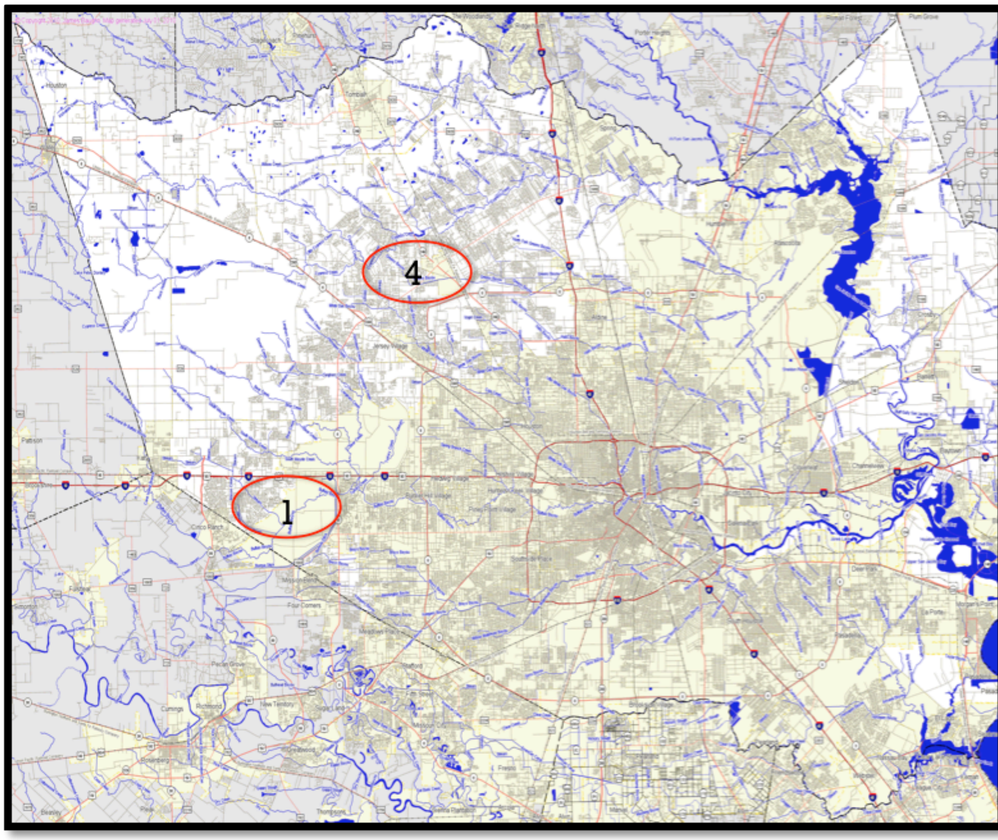


Figure 1: Map of Harris County Waterways, Harris County, Texas {Baughn, 2010 #166}. Red circles are the locations of the study sites, with the number representing the number of streams studied.

Streams were evaluated to determine how the presence of chemicals associated with WWTP facilities might affect the reproductive health of fish and other aquatic organisms. In 2008, the population of Harris County was about 4 million with about 2.2 million living within the city of Houston {United States Census Bureau, 2008 #94}. There are approximately 400 wastewater treatment plants within the metropolitan area that discharge into the bayous (Fig. 2) that ultimately deliver these effluents to Galveston Bay. This study is one of the first to study WWTP effluents in Houston's bayou system although similar research on industrial contaminants has been done in the Houston Ship Channel {Suarez, 2005 #161}.

1.5 Study Organism

The western mosquitofish, *Gambusia affinis*, inhabits freshwater habitats such as creeks, rivers, ponds, and springs {Nelson, 2006 #61}. The mosquitofish is a resilient species that also can inhabit stagnant and polluted water. The species can survive in conditions with low dissolved oxygen, moderate salinity, and moderately low temperatures. As a result, the species has become established in surface waters in many parts of the world where it has been introduced for control of mosquito larvae. *Gambusia affinis* is an excellent model organism for evaluation of reproductive abnormalities due to their sexually dimorphic anal fin and livebearing mode of reproduction (ovoviviparity). Development of a modified anal fin, or gonopodium, in males is dependent on androgens such as testosterone {Grobstein, 1942 #90}. The gonopodium serves as an intromittent organ

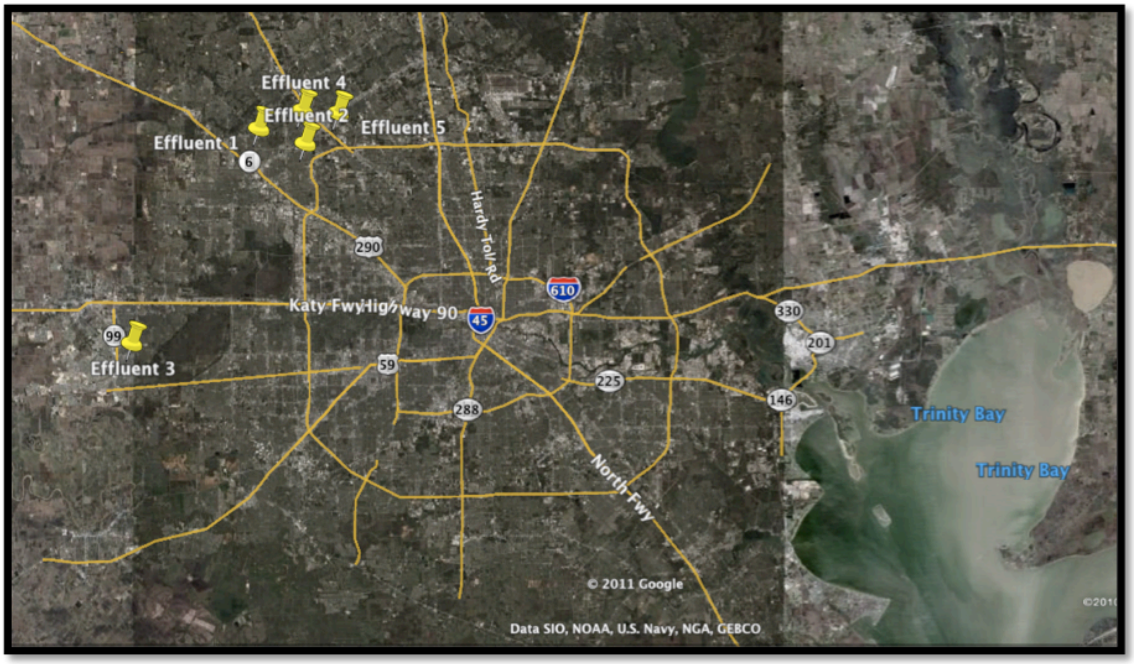


Figure 2: Location study areas in relation to Houston area {Google Inc, 2010 #95}.

for the transfer of sperm during copulation in these live-bearing fish. Previous studies have found that the gonopodium is a good biomarker to use for developmental abnormalities {Batty, 1999 #16;Rawson, 2008 #91}.

Female *Gambusia affinis* can reach a maximum of 70 mm standard length (SL), but males only grow to a maximum length of 40 mm {Krumholz, 1948 #80}. In south Texas, their breeding season occurs from March to October {Thomas, 2007 #56}. Mosquitofish are non-superfetating, which means that they only produce one brood at a time in the ovary, with all of the embryos at the same developmental stage {Turner, 1937 #104}. Females are considered to be gravid when a dark spot appears on each side of their abdomen near the anal fin origin. The age and size at maturation are dependent on the time of year birth {Krumholz, 1948 #80} and some individuals become sexually mature in less than six weeks {Krumholz, 1948 #80}. At the beginning of the breeding season, all of the ova required to produce multiple broods are present in the ovary {Kuntz, 1913 #105}, and females produce multiple broods approximately every 30 days during the spring and summer months {Turner, 1937 #104}. Past studies have found an association between WWTP effluents and abnormalities in mosquitofish {Batty, 1999 #16}. The sensitivity of the gonopodium to small amounts of estrogen as well as previous research establishing an association between WWTP and abnormalities in mosquitofish, make this species an useful model organism for investigation of environmental impacts of domestic wastewater effluents in the bayou system of Harris County.

1.6 Research Objectives

The overall objective of the study was to evaluate evidence of reproductive abnormalities of *Gambusia affinis* in association with WWTP effluents in tributaries of Buffalo Bayou, Greens Bayou, and Cypress Creek in Harris County, Texas, and specifically:

1) to assess evidence of morphological or reproductive abnormalities in mosquitofish downstream from WWTP effluents.
2) to determine abnormalities or lesions in mosquitofish downstream of WWTP effluents.
3) to determine concentration of PPCPs in water samples from below WWTPs effluents.

2. METHODS

2.1 Study Sites and Field Surveys

Five tributaries of Buffalo Bayou, Greens Bayou, and Cypress Creek (Fig. 2) were sampled over a 5-day period during the early summer and late summer (May and August) 2010. Mosquitofish were collected during both periods at 10 sites: 5 sites positioned downstream of a WWTP effluent and 5 sites positioned upstream of these effluents to serve as control sites. Sites were chosen based on accessibility, distance to WWTP, and the absence of any upstream WWTP influence. Treatment sites (B sites, or below WWTP sites) were located 50-200 m downstream from a WWTP effluent. Control sites (A sites, or above WWTP sites) were located approximately 250 m above the same WWTP effluent.

Following the field methods of Franssen {, 2009 #17}, fish were collected with up to 10 seine hauls with a goal of capturing at least 15 adult male and 15 adult female specimens from each site. Fish were anesthetized using tricaine methanesulfonate (MS-222) following Texas A&M Animal Use Protocol 2005-117. Specimens were then preserved in 10% formalin for morphological and histological analyses. Specimens were later transferred to 70% ethanol for long-term storage.

At each survey site, environmental variables, including dissolved oxygen (DO), water temperature and conductivity, were measured with a YSI model 85 meter, and pH was measured with a pH Testr20 electronic handheld pH meter. The distance to the wastewater treatment plant and habitat conditions, such as surrounding riparian vegetation and type of land use, within 20 m of waterway was recorded.

2.2 Morphological and Reproductive Indicators

For each collected specimen, SL was measured to the nearest 0.05 mm. The gonads of both males and females were removed and weighed (dry weight) to calculate gonadosomatic index (GSI). The dry weights of the liver and eviscerated body of the fish were determined to the nearest 10^{-4} g. In male specimens, gonopodial indices such as gonopodium length/body length ratio as well as the percentage of male fish with fully-developed hooks on the gonopodium were recorded {Game, 2006 #86}. Embryo size and clutch size were measured in female specimens. Ovary and embryo developmental stages were recorded following the techniques for poecilid development of Haynes {, 1995 #92} and Meffe {, 1987 #100}. Total dry weight of the brood was measured to the nearest 0.1 mg {Kristensen, 2007 #88}.

2.3 Histological Examination

Eighteen specimens were randomly chosen for preparation at the Texas A&M University College of Veterinary Medicine Histopathology Lab. A random subsample of 5-8 adult specimens was chosen from each study site for histological examination of gonads. The sex of adult size classes was determined based on the presence of the male gonopodium, and presence of a gravid spot near the base of the anal fin in females. The entire gonad of each specimen was fixed in 10% formalin, embedded in paraffin wax, sectioned at 9-12 μm and stained with hematoxylin and eosin for examination under a light microscope. Cross-sections of each gonad were analyzed for evidence of abnormalities such as the presence of ovarian tissue in testicular tissue and the presence of underdeveloped gonads.

2.4 Chemical Analysis

Ten water samples were collected August 2011 in acetone washed amber bottles. Samples were then immediately stored on ice and were analyzed at the Environmental Sciences Laboratory at Baylor University within 24 hours. Samples were extracted with HLB glass cartridges from Waters Corporation. All extractions were performed on an Auto Trace automated SPE system. The SPE cartridges were preconditioned with MTBE, methanol, and nanopure water. The samples were loaded onto cartridges and were air-dried and eluted with methanol followed by 5 mL of 10/90 (v/v) MeOH/MTBE into culture test tubes. The extract was concentrated with nitrogen to dryness and then brought to a final volume of 1 mL using 5:95 MeOH: HCOOH (0.1%). Prior to analysis, samples were sonicated for 1 min and filtered using Pall Acrodisc hydrophobic Teflon Supor membrane syringe filters (13-mm diameter; 0.2-μm pore size).

Analytes were analyzed with Extend-C18 column (Agilent Technologies, Palo Alto, CA) connected with an Extend-C18 guard cartridge 12.5 mm x 2.1 mm (5 μm, 80 Å) (Agilent Technologies, Palo Alto, CA) with a Varian ProStar pump system equipped with a Model 410 autosampler. Additional chromatographic parameters were as follows: injection volume, 10 μL; column temperature, 30 ºC; flow rate, 350 μL/min. Eluted analytes were monitored by MS/MS using a Varian model 1200L triple-quadruple mass analyzer equipped with an electrospray interface (ESI).

An isotopic labeled version of each analyte, corresponding to the isotopes added to each sample prior to extraction, was added to each calibration point at a concentration of 100 µg/L to generate a relative response ratio. Recoveries of the isotopes were compared with the relative response ratio and a concentration for the unlabeled analyte was calculated. Linear or quadratic regression $r^2 \geq 0.998$ was used for all analytes. Instrument calibration was monitored through the use of continuing calibration verification (CCV) samples with an acceptability criterion of ±20%.

2.5 Statistical Analysis

All statistical analyses were performed using JMP (version 8). A two-way ANOVA was used to compare differences above and below WWTP by streams as well as by date. For data that were not normally distributed, (HSI, GSI, average egg size, average egg/embryo weight and fecundity), the nonparametric Mann-Whitney U-test was used to test for differences between position relative to the WWTP (A, B), stream (1-5), and survey period (May, August).

Gonopodium length, gonad weight and liver weight were compared between treatment and control sites across all streams combined and also for individual streams using ANCOVA. Standard length and carcass weight were used as covariates to correct for differences in body size for gonopodium length and gonad and liver weights. Gonad, liver and carcass weight were logarithmically transformed to improve normality and homogeneity of variance. ANCOVA was run with a stream position x covariate interaction term to confirm homogeneity of slope, and when confirmed, the test was rerun without the interaction term.

Since specimens were collected during different periods of the summer (May and August), statistical comparisons were performed with and without survey period as an additional independent variable. Water quality variables such as pH and conductivity were analyzed to evaluate differences above and below the WWTP effluents. Bonferroni adjustments for multiple comparisons of datasets for females (α'=0.001) and males (α'=0.0025) were used to infer significant differences among pair-wise comparisons.

3. RESULTS

3.1 Physiochemical Properties

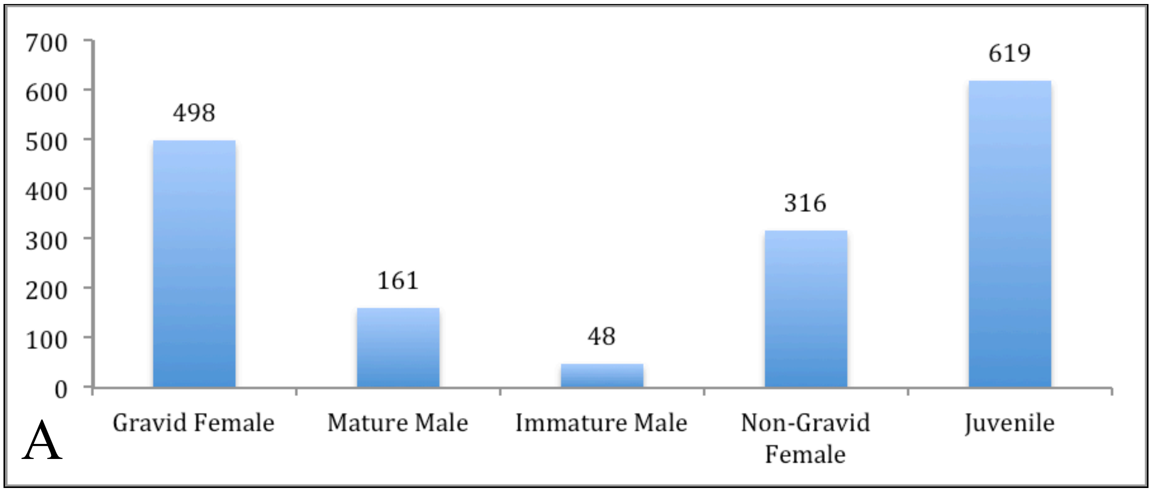
None of the water quality parameters (Table 1) showed a significant difference between sites located above and below the WWTP ($p > 0.05$, ANOVA). Temperature ranged from 25.8-36.0 and pH from 7.8-9.0. Dissolved oxygen, conductivity, and salinity varied greatly between dates. In May, DO ranged from 3.79 to 16.6 mg/L, and in August DO ranged from 1.05 to 13.06 mg/L. The highest salinity value (0.7 ppt) was measured in August. Conductivity values ranged from 147-1060 µS and 9.4-1509 µS for May and August, respectively. Substrate types present at the study sites included mud/clay, sand, silt, gravel and concrete with the most common being mud/clay. At all sites, the riparian zone consisted of mowed grass or shrubs. Average stream depth ranged from 0.12 meters to 0.51 meters.

3.2 Fish Abundance and Sex Ratios

A total of 3,359 specimens was collected; 1,642 were collected during the May survey, of which 498 were gravid females, 161 were mature males, 48 were immature males, 316 were non-gravid females, and 619 were juveniles (Figure 3a). During the August survey, 1,717 specimens were collected, including 264 gravid females, 63 mature males, 553 non-gravid females, 32 immature males, and 1164 juveniles (Figure 3b). The overall proportion of males to females was 0.235. The values for the sex ratios by survey site and date are given in Table 2. Sex ratio did not differ between sites above and below WWTPs ($p > 0.45$, ANOVA) or by survey period ($p > 0.76$, ANOVA).

Table 1. P-values from comparisons (Mann Whitney U-test) of water quality parameters measured at sites above and below WWTP for data grouped across all streams and survey periods.

Stream	pH	Temperature	Dissolved O_z (mg/L)	Conductivity (µS)	Salinity (ppt)
1	0.69	0.42	0.75	0.54	1.00
2	1.00	0.90	0.65	0.10	0.70
3	0.59	0.66	0.35	0.97	1.00
4	0.87	0.94	0.91	0.93	0.53
5	0.34	0.91	0.71	0.98	1.00



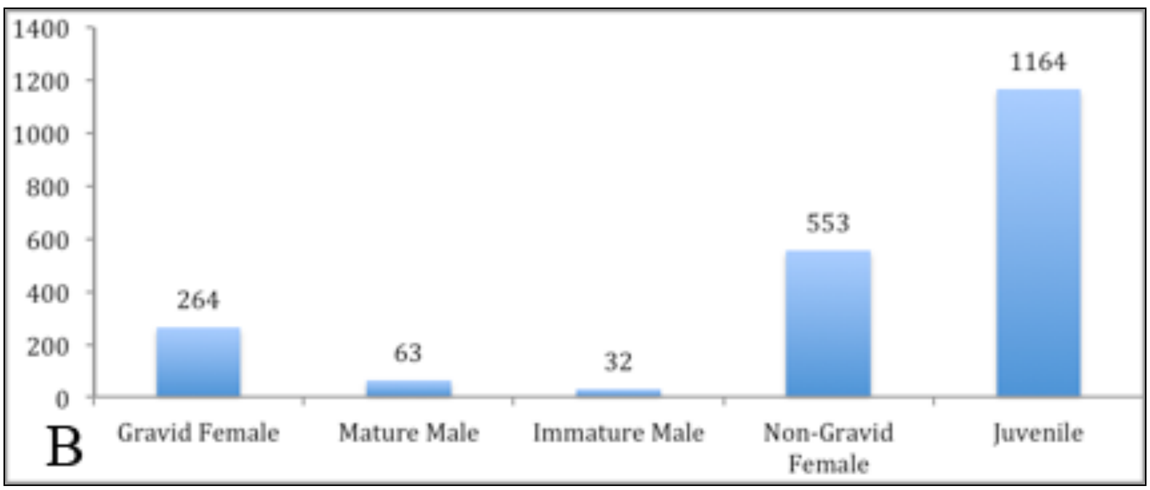


Figure 3. Number of fish captured in A) May and B) August.

Table 2. Male:female sex ratio by survey period, stream, and position.

Period	Stream	Position	Sex Ratio
May	1	Above	0.261
May	1	Below	0.228
May	2	Above	0.193
May	2	Below	0.217
May	3	Above	0.241
May	3	Below	0.205
May	4	Above	0.270
May	4	Below	0.292
May	5	Above	0.080
May	5	Below	0.009
Aug	1	Above	0.163
Aug	1	Below	0.289
Aug	2	Above	0.040
Aug	2	Below	0.156
Aug	3	Above	0.169
Aug	3	Below	0.118
Aug	4	Above	0.124
Aug	4	Below	0.130
Aug	5	Above	0.133
Aug	5	Below	0.333

3.3 Female Reproductive and Morphological Parameters

Only those comparisons that were statistically significant are presented as figures in the Results section; all other comparisons were non-significant. Overall, there were no statistically significant differences in standard length of mature females above and below the WWTP when date was considered ($p > 0.001$, ANOVA, Tables 3a and 3b). Female SL ranged from 18-29 mm above WWTPs and 18-39 mm below WWTPs. Females found above WWTPs tended to be larger (23.8 mm) than those found the below WWTPs (23.3 mm) but not significantly ($p > 0.001$, ANOVA). When sites above and below WWTP outflows were compared by survey periods separately (Tables 3a and 3b), no significant differences in standard length were observed for females.

Female GSI values were not significantly different for above and below WWTP ($p > 0.001$, Mann-Whitney U-test). Average female GSI above the WWTP was 19.9% and below the WWTP was 17.1% ($X^2 = 0.005$, $p > 0.94$, Mann-Whitney U-test). When position was compared among streams, there were no significant differences when compared to the Bonferroni corrections (Tables 4a and 4b). When position relative to WWTP was compared by position for HSI, there were no significant differences seen for females ($p > 0.05$, Mann-Whitney U-test). Above the WWTP, the average HSI was 2.34% for females and below the WWTP for female HSI was 2.25%. When position was compared among streams, no significant differences were seen for females (Tables 4a and 4b).

Table 3a: ANOVA results of female parameters for each stream during May. n and p-value refer to comparison of sites above the WWTP versus below the WWTP. Degrees of freedom for all comparisons is 1.

	Stream 1		Stream 2		Stream 3		Stream 4		Stream 5	
Variables	n	*p*-value	n	*p*-value	n	*p*-value	n	*p*-value	n	*p*-value
SL	75	0.006	302	0.0018	53	0.98	138	0.008	248	0.96
GSI	20	0.24	20	0.31	19	0.26	17	0.77	20	0.02
HSI	20	0.11	20	0.14	20	0.55	20	0.7	20	0.11
Mean fecundity	6	0.42	5	0.47	11	0.95	4	0.72	5	0.9
Mean # of oocytes	6	0.26	5	0.4	11	0.46	4	0.74	5	0.81
Mean # of embryos	14	0.04	14	0.047	9	0.5	5	0.53	14	0.006
Egg/weight	6	0.67	5	0.37	11	0.61	4	0.0066	5	0.0013
Embryo/weight	14	0.13	14	0.87	5	0.12	5	0.57	12	0.6
Average egg size	6	0.67	5	0.37	11	0.61	4	0.0066	5	0.013
Average egg/embryo stage	20	0.07	19	0.79	20	0.26	9	0.11	19	0.27

Table 3b. ANOVA results of female parameters for each stream during August. n and p-value refer to comparison of sites above the WWTP versus below the WWTP. Degrees of freedom for all comparisons is 1. No data available for comparison is listed as n.d.

Variables	Stream 1		Stream 2		Stream 3		Stream 4		Stream 5	
	n	*p* value	n	*p* value	n	*p* value	n	*p* value	n	*p*value
SL	122	0.02	51	0.5	124	0.91	146	0.67	17	0.15
GSI	20	0.0003	20	0.0017	19	0.43	16	0.02	9	0.31
HSI	20	0.38	20	0.51	20	0.09	20	0.03	9	0.04
Mean fecundity	4	0.01	7	0.29	7	0.15	n.d.	n.d.	n.d.	n.d.
Mean # of oocytes	4	0.01	7	0.32	7	0.22	n.d.	n.d.	n.d.	n.d.
Mean # of embryos	9	0.04	12	0.96	11	0.06	n.d.	n.d.	n.d.	n.d.
Egg/weight	4	0.93	7	0.71	7	0.11	n.d.	n.d.	n.d.	n.d.
Embryo/ weight	8	0.6	12	0.27	8	0.01	n.d.	n.d.	n.d.	n.d.
Average egg Size	4	0.93	7	0.71	7	0.11	n.d.	n.d.	n.d.	n.d.
Average egg/embryo stage	11	0.05	19	0.38	15	0.78	8	0.05	n.d.	n.d.

Table 4a: Mann-Whitney U-test results for females parameters by stream in May. Comparisons are for sites above the WWTP versus below WWTPs in each stream. Bonferroni corrected *p* =0.001 and degrees of freedom for all comparisons is 1.

Variables	Stream 1		Stream 2		Stream 3		Stream 4		Stream 5	
	X^2	*p* value	X^2	*p* value	X^2	*p* value	X^2	*p* value	X^2	*p* value
GSI	1.12	0.29	0.97	0.33	0.11	0.74	0.09	0.77	4.81	0.03
HSI	1.85	0.17	3.29	0.07	0.97	0.33	3.02	0.08	2.77	0.10
Mean fecundity	0.86	0.35	1.33	0.25	0.3	0.58	0.2	0.65	0	1
Mean # of oocytes	1.93	0.16	0.79	0.37	0.31	0.58	0.2	0.65	0.09	0.77
Mean # of embryos	3.76	0.05	9.33	0.002	0.02	0.9	0	1	7.95	0.005
Egg/weight	0.21	0.64	0.33	0.56	0.3	0.58	1.8	0.18	3	0.08
Embryo/ weight	1.35	0.25	0.42	0.52	3	0.08	0	1	0.16	0.68
Average egg/embryo stage	3.53	0.06	0.002	0.97	1.26	0.26	2.4	0.12	1.17	0.28

Table 4b: Mann-Whitney U-test results for female parameters by stream in May. Comparisons are between sites above the WWTP versus below WWTPs in each stream. Bonferroni corrected *p* =0.001 and degrees of freedom for all comparisons is 1. No data available for comparison is listed as n.d.

Variables	Stream 1		Stream 2		Stream 3		Stream 4		Stream 5	
	X^2	*p* value	X^2	*p* value	X^2	*p* value	X^2	*p* value	X^2	*p* value
GSI	10.08	0.0015	9.14	0.003	0.33	0.57	3.81	0.05	0.60	0.44
HSI	1.29	0.26	0.09	0.76	2.52	0.11	5.49	0.02	2.40	0.12
Mean fecundity	1.8	0.18	1.35	0.25	2	0.16	n.d	n.d	n.d	n.d
Mean # of oocytes	1.8	0.18	0.95	0.33	0.8	0.37	n.d	n.d	n.d	n.d
Mean # of embryos	4.27	0.04	0.03	0.86	1.69	0.19	n.d	n.d	n.d	n.d
Egg/weight	0.2	0.65	0.15	0.7	4.5	0.03	n.d	n.d	n.d	n.d
Embryo/ weight	0.43	0.51	0.72	0.4	5	0.03	n.d	n.d	n.d	n.d
Average egg/embryo stage	2.86	0.09	0.44	0.51	0	1	2.48	0.12	n.d	n.d

Weights of liver or gonad versus body weight were not different between females from above and below WWTPs. When data were analyzed for survey periods separately, liver weight did not differ between sites above and below WWTPs ($p > 0.05$, ANCOVA, Table 5a). When data were analyzed by separate survey periods, there were no significant inter-stream differences for female gonad weight (Table 5b).

Mean fecundity was not significantly different above and below WWTPs when data were analyzed for all streams combined for separate survey periods, or when data were separated by individual streams and survey periods ($p > 0.59$, Mann-Whitney U-test, Tables 4a and 4b). The number of unfertilized oocytes, relative to position above or below a WWTP also was not significantly different among streams ($p < 0.05$, Mann-Whitney U-test, Tables 4a and 4b), whether data were grouped across both survey periods or compared within each survey period separately. The number of embryos was different between sites above and below WWTPs ($p < 0.0004$, Mann-Whitney U-test) for each survey period when analyzed separately and data were grouped across streams. In August, females below WWTPs had an average of 10.92 embryos compared to 5.70 embryos for females above WWTPs (Figure 4). The number of embryos, however, was not significantly different above and below WWTPs among streams during either survey period ($p > 0.0077$, Mann-Whitney U-test, Tables 4a and 4b).

Table 5a: ANCOVA results for comparison of liver weights (with carcass weight as the covariate) for females above and below WWTP by separate streams and survey periods.

May

Streams	F	df	*p* value
1	4.48	1,19	0.05
2	2.65	1,19	0.122
3	0.27	1,19	0.61
4	2.95	1,18	0.11
5	1.91	1.19	0.19

August

Streams	F	df	*p* value
1	0.007	1,19	0.93
2	0.54	1,19	0.47
3	2.59	1,19	0.13
4	1.93	1,19	0.18
5	1.52	1,9	0.26

Table 5b: ANCOVA results for comparison of ovary weights (with carcass weight as the covariate) for females above and below WWTP by separate streams and survey periods. No data available for comparison is listed as n.d.

May

Streams	F	df	*p* value
1	0.2	1,19	0.66
2	0.22	1,19	0.65
3	0.45	1,16	0.52
4	0.19	1,16	0.67
5	5.45	1,19	0.03

August

Streams	F	df	*p* value
1	6.43	1,19	0.02
2	11.9	1,19	0.0031
3	0.51	1,17	0.49
4	4.19	1,14	0.07
5	n.d.	0,5	n.d.

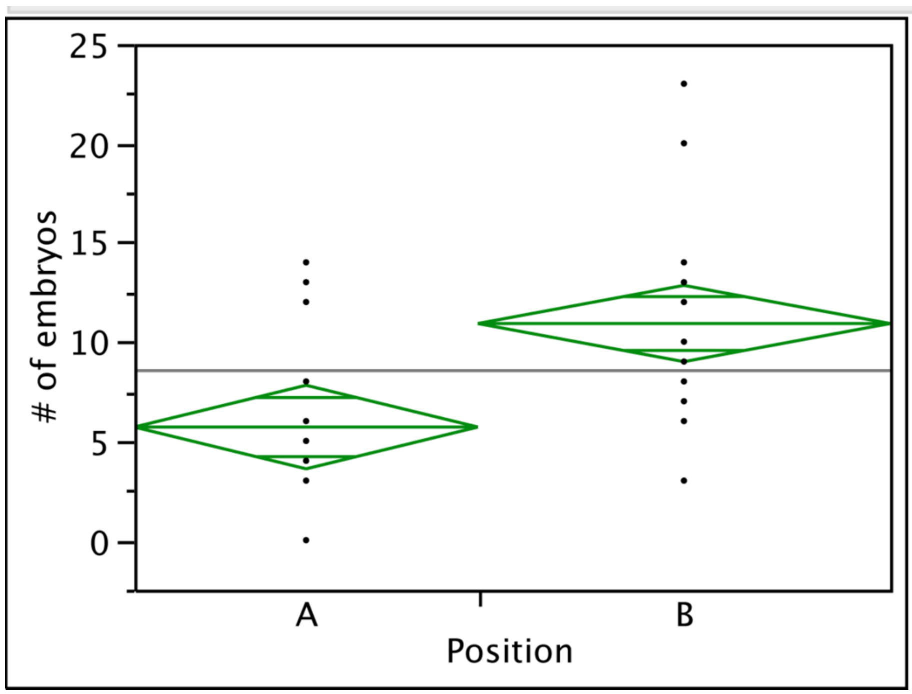


Figure 4. Number of embryos by position above (A) or below (B) WWTP for all streams during August.

No significant difference in mean egg weight was obtained when sites above and below WWTP were compared for individual streams and survey periods ($p > 0.04$, Mann-Whitney U-test, Tables 3a and 3b). Embryo weight was not significantly different between sites above and below WWTPs when data were combined across streams and both survey periods, or when streams and survey periods were examined separately ($p < 0.05$, Mann-Whitney U-test, Tables 3a and 3b). The stage of development for embryos was not significantly different between sites above and below WWTPs when separated by survey period and stream ($p > 0.05$, Mann-Whitney U-test).

3.4 Male Reproductive and Morphological Parameters

No statistically significant differences were observed in standard length of mature males above and below the WWTP when date was considered ($p > 0.001$, ANOVA, Table 6). Males collected above WWTPs ranged from 18-25 mm and those below were between 18-25.7 mm. When compared by survey periods, sites above and below WWTP effluents were not significantly different in standard length (Table 6).

GSI values were not significantly different above and below WWTP ($p > 0.05$, Mann-Whitney U-test). Male GSI values 1.97% above the WWTP and 1.7% below the WWTP ($X^2 = 0.09$, $p > 0.76$, Mann-Whitney U-test). Males in stream 2 collected during May males collected during May had a significantly different GSI values between positions ($X^2 = 9.22$, $p < 0.0024$, Mann-Whitney U-test, Table 7). When position was compared among streams, there were no significant differences (Table 7).

Table 6: ANOVA results of male parameters for each stream and survey period. n and p-value refer to comparisons of sites above versus below WWTPs. Degrees of freedom for all variables is 1. Significant results (α'= 0.0025) are indicated with an asterisk. No data available for comparison is listed as n.d.

May

Variables	Stream 1		Stream 2		Stream 3		Stream 4		Stream 5	
	n	*p* value	n	*p* value	n	*p* value	n	*p* value	n	*p* value
SL	25	0.29	74	0.006	15	0.97	53	0.21	42	0.97
GSI	16	0.02	18	0.33	12	0.59	18	0.67	16	0.16
HSI	16	0.41	18	0.59	12	0.15	18	0.35	16	0.88
Gonopodium Length	25	0.23	74	0.92	15	0.83	53	0.24	42	0.2943

August

Variables	Stream 1		Stream 2		Stream 3		Stream 4		Stream 5	
	n	*p* value	n	*p* value	n	*p* value	n	*p* value	n	*p*-value
SL	41	0.22	6	0.96	21	0.69	21	0.13	4	0.93
GSI	11	0.53	n.d.	n.d.	11	0.29	14	0.43	4	0.17
HSI	11	0.12	n.d.	n.d.	11	0.37	14	0.07	4	0.03
Gonopodium Length	41	0.1	6	0.0013*	21	0.02	21	0.36	4	0.76

Table 7: Mann-Whitney-U test results for males by stream and survey period. Comparisons are between sites above versus below WWTPs for each stream. Bonferroni corrected *p*= 0.0025 with significant results (*) and degrees of freedom= 1. No data available for comparison is listed as n.d.

May

Variables	Stream 1		Stream 2		Stream 3		Stream 4		Stream 5	
	X^2	*p* value	X^2	*p* value	X^2	*p* value	X^2	*p* value	X^2	*p* value
GSI	9.22	0.0024*	0.39	0.53	0.03	0.87	0.79	0.37	1.86	0.17
HSI	0	1	0.008	0.93	2.56	0.11	0.72	0.4	0.07	0.79

August

Variables	Stream 1		Stream 2		Stream 3		Stream 4		Stream 5	
	X^2	*p* value	X^2	*p* value	X^2		X^2	*p* value	X^2	*p* value
GSI	0.22	0.64	n.d.	n.d.	1.29	0.26	0.36	0.55	2.4	0.12
HSI	3.56	0.06	n.d.	n.d.	0.89	0.34	6.08	0.01	2.4	0.12

For HSI, there were no significant differences seen for males ($p > 0.05$, Mann-Whitney U-test) with above the WWTP value 0.35% for males and below the WWTP male HSI was 0.33%. Position was compared among streams, and no significant differences were seen for males (Table 7).

Liver or gonad versus body weight was not different above and below WWTPs. Liver weight by survey period was not different for between sites above and below WWTPs ($p > 0.05$, ANCOVA, Table 8a). Neither position relative to WWTP nor stream had a significant influence on male liver weight, even after accounting for temporal variation (Table 8a). When the dataset was divided by survey periods, testes weight showed a significant difference above and below the WWTP during May in stream 2 ($p < 0.0038$, ANCOVA, Table 8b) but this comparison was not significant during August ($p > 0.0025$, ANCOVA, Table 8b).

Mean gonopodium length did not differ above and below WWTPs when data were grouped across streams and survey periods, or when survey periods were examined separately ($p > 0.05$, ANCOVA). The mean gonopodium length of males above WWTPs was 6.83 mm, and gonopodium length was 6.71 mm for males below WWTPs. Even though not statistically significant, males captured in May had a longer gonopodium (mean above WWTP= 6.92 mm, below= 6.77 mm) compared to males captured during August (mean above WWTP= 6.52 mm, below= 6.63 mm)(Table 6). When data were separated by stream and survey period, no significant differences in mean gonopodium length were found above versus below WWTPs (Table 9).

Table 8a: ANCOVA results for comparison of liver weights (with carcass weight as the covariate) for males above and below WWTP by separate streams and survey periods. No data available for comparison is listed as n.d.

May			
Streams	F	df	*p* value
1	0.23	1,10	0.64
2	0.12	1,8	0.74
3	4.84	1,8	0.07
4	0.0001	1,4	0.99
5	0.03	1,6	0.86

August			
Streams	F	df	*p* value
1	10.18	1,8	0.02
2	n.d.	0,4	n.d.
3	1.6	1,10	0.24
4	4.13	1,9	0.08
5	25.71	1,3	0.12

Table 8b**:** ANCOVA results for comparison of testes weights (with carcass weight as the covariate) for males above and below WWTP by separate streams and survey periods. Bonferroni corrected p-value= 0.0025 with significant results (*) and degrees of freedom= 1. No data available for comparison is listed as n.d.

May			
Streams	F	df	*p* value
1	12.76	1,14	0.0038*
2	0.03	1,15	0.87
3	0.28	1,10	0.61
4	0.36	1,12	0.56
5	1.23	1,14	0.29

August			
Streams	F	df	*p* value
1	4.97	1,6	0.09
2	n.d.	0,4	n.d.
3	0.86	1,10	0.38
4	0.86	1,12	0.38
5	3.22	1,3	0.32

Table 9: ANCOVA results for comparison of gonopodium (with standard length as the covariate) for males above and below WWTP by separate streams and survey periods. No data available for comparison is listed as n.d.

May			
Streams	F	df	p-value
1	0.35	1,20	0.56
2	0.94	1,52	0.34
3	0.007	1,10	0.93
4	0.45	1,35	0.51
5	0.13	1,31	0.72

August			
Streams	F	df	p-value
1	0.49	1,17	0.5
2	n.d.	1,4	n.d.
3	7.11	1,15	0.019
4	0.8	1,17	0.38
5	0.1	1,3	0.8012

3.5 Histopathology

Results of the examination of fish specimens are shown in Table 10. No evidence of intersex (ovarian and testicular tissue in the same individual) was found in any of the specimens examined. All but one of the male mosquitofish specimens showed evidence of active spermatogenesis, which is expected of a mature male during summer. In ovaries, stages of follicular development ranged from no ovarian follicular activity to very large follicles. The most obvious anomaly was the presence of parasites in the gills, body cavity, intestine, muscle and cartilage. Parasitic flatworms (Trematoda) were the most conspicuous parasites found in the body cavity. Tapeworms were found in the intestines of two specimens, and intramuscular trematodes were found in two other specimens; both types of parasites seemed not to adversely impact the individuals' health. One specimen was found with mxyospores within cartilage (Table 10). Evidence of inflammation and lesions were seen in several specimens, however specific causal agents could not be identified.

3.6 Water Chemical Analysis

Analysis of water samples from above and below WWTPs revealed a wide variety of pharmaceuticals and other compounds. Caffeine and sucralose were the most prevalent compounds found in the samples (found in all samples), and erythromycin and celecoxib were the least prevalent compounds found (2 out of 10 samples) (Table 11). The most abundant compound was caffeine, with a maximum concentration of 1100 ηg/L, and the least abundant compound was methylphenidate at around 0.52 ηg/L.

Table 10: Summary of histology analysis.
*nml= no microscopic lesions, +ne= not examined

Specimen	Brain	Skin	Gills	Stomach	Small intestine	Swim bladder	Spleen
1	heterophils present	No tail fin	parasite present	nml*	nml	nml	ne
2		nml	nml	nml	nml	nml	nml
3	heterophils present	Thickening and heterophils	nml	nml	nml	nml	nml
4	nml	minor thickening	parasite present	nml	nml	nml	ne+
5	heterophils present	nml	nml	nml	nml	nml	nml
6	nml	nml	nml	nml	nml	nml	ne
7	nml	nml	nml	nml	nml	nml	ne
8	nml	Thickening and heterophils	nml	nml	cestode	nml	ne
9	heterophils present	nml	parasite present	nml	nml	nml	ne
10	heterophils present	Thickening and heterophils	parasite present	nml	nml	nml	ne
11	nml	nml	multiple parasites	nml	nml	nml	ne
12	nml	nml	parasite present	nml	nml	parasites present	heamosiderosis
13	nml	nml	nml	nml	nml	nml	ne
14	nml	nml	nml	nml	ne	nml	ne
15	nml	nml	nml	cestode	nml	nml	ne
16	heterophils present	nml	nml	nml	nml	nml	heterophils present
17	heterophils present	nml	nml	nml	nml	nml	haemosiderosis
18	heterophils present	nml	parasite present	nml	nml	nml	haemosiderosis

Table 10.-Continued

Specimen	Pancreas	Liver	Sex	Reproduction	Muscle	Bone
1	nml	no glycogen	female	large follicles	nml	nml
2	nml	glycogen	female	small follicles	nml	nml
3	nml	glycogen	male	active spermatogenesis	nml	nml
4	nml	glycogen	female	very large follicles	nml	nml
5	nml	glycogen	male	no spermatogenesis		nml
6	nml	glycogen	gonads not visible	not observed	nml	nml
7	nml	glycogen	male	active spermatogenesis	nml	nml
8	nml	no glycogen	female	very large follicles	nml	parasites in cartilage
9	nml	glycogen	female	Inactive	heterophil and muscle degeneration	nml
10	nml	glycogen	female	very large follicles	nml	nml
11	nml	parasite present	male	active spermatogenesis	nml	nml
12	nml	3 large parasites	female	large follicles	nml	nml
13	nodule present	large parasite present; glycogen	female	very, very large follicles.	nml	nml
14	nml	ne	male	active spermatogenesis	nml	nml
15	nml	glycogen	female	small follicles	nml	nml
16	nml	glycogen, plus a single heterophilic nodule	female	ovary with no activity	nml	nml
17	Increased infiltration with heterophils	multiple parasites present, glycogen present	gonads not visible	not observed	muscle degeneration and heterophils present	
18	nml	parasites; little glycogen present	male	active spermatogenesis	parasite present	

Samples collected above the WWTP had lower concentrations for 5 out of the 18 compounds tested plus 9 non-detects among the 18 measured compounds. Sucralose, sulfamethoxazole, diphenhydramine, dilitiazem, and gemfribrozil were lower in water samples taken above one WWTP compared to samples taken below the other WWTPs (Table 11).

Table 11. Eighteen pharmaceuticals analyzed in bayou samples (for streams 1-4, sites were below WWTP; for stream 5, the site was above the WWTP). Concentrations are in ng/L. ND=nondetectable

		Sites Below WWTP								Site Above WWTP	
Analyte	Use	Stream -1(1)	Stream -1(2)	Stream –2(1)	Stream -2(2)	Stream -3(1)	Stream -3(2)	Stream - 4(1)	Stream -4(2)	Stream-5(1)	Stream-5(2)
Caffeine	stimulant	1100	1010	43	40	52	41	41	39	220	53
Sucralose	artificial sweetener	690	570	820	950	1100	950	850	800	180	260
Sulfamethoxazole	antibiotic	890	920	850	880	1300	1400	20	17	4.7	5.0
Diltiazem	antihypertensive	1.8	1.9	15	17	34	38	4.4	3.7	0.82	0.83
Diphenhydramine	allergy	8.6	7	47	46	60	55	5.8	5.6	0.57	0.64
Acetaminophen	fever reducer	ND	ND	ND	ND	10	10	11	14	30	32
Atenolol	high blood pressure	39	50	100	100	130	140	ND	ND	3.9	3.7
Codeine	analgesic	100	88	6.9	8.0	ND	ND	ND	ND	ND	ND
Trimethoprim	antibiotic	47	39	17	18	45	46	ND	ND	ND	ND
Methylphenidate	ADHD	ND	ND	0.52	0.60	0.88	1.2	ND	ND	ND	ND
Propranolol	migraine	ND	ND	20	20	20	23	ND	ND	ND	ND
Carbamazepine	antiseizure	ND	ND	220	220	140	140	150	160	11	11
Erythromycin	antibiotic	ND	ND	ND	ND	34	29	ND	ND	ND	ND
Diazepam	anti-anxiety	ND	ND	1.0	0.83	1.0	0.87	2.0	ND	ND	ND
Warfarin	anticoagulant	13	12	1.0	1.0	1.2	1.6	ND	ND	ND	ND
Celecoxib	arthritis medicine	160	120	ND	ND	ND	ND	ND	ND	ND	ND
Diclofenac	arthritis	47	40	17	16	35	39	ND	ND	ND	ND
Gemfibrozil	antihyperlipidemic	ND	ND	140	140	46	50	6.3	8.1	1.9	2.4

4. DISCUSSION AND CONCLUSION

4.1 Discussion

Overall, chemicals being discharged from the WWTP effluents do not appear to be negatively impacting mosquitofish in the bayous of western Harris County. PPCPs were found in water samples collected below treatment plants, but at lower concentrations than those reported to cause problems in fish {Fent, 2006 #151;Winter, 2008 #150}. However, in this study, sampling was focused on the most upstream WWTPs in the bayous where lowest concentrations of PPCPs would be expected. This study did not examine the effect of multiple treatment facilities on one stream system. Sampling of sites located further downstream, where fish would be subjected to effluents from multiple WWTPs, might yield different results.

Mosquitofish populations are typically female biased {Krumholz, 1948 #80}, and this study determined that sex ratios were not significantly skewed from this pattern in any of the five streams. Even though not statistically significant, females and males collected below WWTPs were smaller than those above WWTPs. This trend might not represent a response to chemicals in WWTP effluent, and instead could be due to natural variation found among fish populations. Differences in body size also can represent a response to differential predation pressure {Britton, 1982 #157}. Similarly, studies have found that temperatures below WWTPs tend to be warmer compared to upstream reaches {Gower, 1978 #154}. Although body length differed above and below WWTP for individual streams, there were no consistent patterns.

The balance of evidence from this study suggests that chemicals in WWTP effluents have not greatly impacted liver or gonadal development in mosquitofish. HSI for both sexes were not significantly different when compared by position or by date. Similarly, GSI values obtained in this study were within the range of values reported for mosquitofish in other field studies {Park, 2006 #137}. Fluctuations in environmental conditions, such as water quality and productivity, as well as the presence of egg or embryos in females, can influence GSI in live bearing fishes {Edwards, 2005 #138}. Non-significant differences in liver and gonad weights above and below WWTPs were inconsistent and difficult to interpret in the context of chemical pollutants. This was similar to Angus' {, 2002 #1} findings and could indicate either an absence or low concentrations of estrogenic or anti-androgenic compounds. However, further chemical and biological analyses, with greater spatial and temporal coverage, are needed before extending this conclusion broadly to the Houston bayou system in general.

None of the female parameters that were measured were significantly different when position relative to the WWTP was compared among streams. The average number of eyed embryos in ovaries of females captured above WWTPs was larger (but not significantly) in the August collection, which could be due to natural variation in size since larger females have been shown to produce more offspring {Krumholz, 1948 #80}. Another potential explanation for observed fecundity differences is the presence of a trade-of between production of many smaller eggs and few larger eggs as a response to environmental contaminants {Kime, 1998 #59} or other environmental factors {Reznick, 1989 #162}. Krumholz (1948){, #80;, 1948 #80} found that time of birth in the season can limit the number of broods; offspring born early in the breeding season will mature in time to achieve 2-3 broods before winter, whereas those born later and successfully overwintering will mature during their second summer and have an opportunity to produce 4-5 broods during their lifetime {Krumholz, 1948 #80}. In mosquitofish, subsequent broods often have fewer embryos than previous ones and temporally dynamic factors, such as natural abiotic environmental conditions, biotic interactions, population density, and mating frequency, could explain brood size changes as the breeding season progresses {Krumholz, 1948 #80}. There were fewer gravid females during August, which is expected because it was later in the breeding season. However, since sampling was only done in one year (2010), all conclusions made are speculative and multiple sample collections are needed to make a definitive conclusion.

Previous research {Batty, 1999 #16;Rawson, 2008 #91} found that male mosquitofish below WWTPs had significantly shorter gonopodia than those from sites not receiving WWTP effluents. In this study, this phenomenon was not observed, and gonopodial length did not differ significantly when males captured above and below WWTPs were compared. Even though stream 2 in August showed a difference when gonopodium length was corrected for body size, this was not statistically significant. Given that there were no consistent patterns in mean gonopodium length in relation to WWTPs, differences in gonopodia likely are due to natural variation rather than effect of contaminants. Angus {, 2002 #1} found that males exposed to effluent did not have shorter gonopodia nor were any detectable levels of vitellogenin found, the latter being an indicator of exposure to estrogenic compounds. Previous research has found the gonopodium to be a good bioindicator for chemical exposure {Doyle, 2002 #127;Game, 2006 #86;Rawson, 2008 #91}. The fact that no significant differences were found in this study suggests that the concentrations of chemicals found in the bayou samples appear not to cause an effect on the development of the organ in mosquitofish.

Even though the mosquitofish has been used to identify negative impacts of WWTP effluents in other ecotoxicological studies {Rawson, 2008 #91;Doyle, 2005 #83;Game, 2006 #86}, it is not as widely used as the fathead minnow or the Japanese medaka. This limits comparisons to other fish studies. A limitation of using mosquitofish is the fact that they are short lived; it is rare that individuals live longer than a year. A short lifespan could limit chronic effects of chemical exposure as well as acute effects, such as death, that would not be detected in this field study. The liver and gonads of mosquitofish are so small that my electronic balance was not able to detect any differences in wet versus dry weights of some of the smallest samples. If minute differences in dry weight had actually occurred between different populations, our equipment was not sensitive enough to measure them.

Histopathology revealed no evidence of somatic or reproductive abnormalities in specimens examined in this study. The presence of ovo-testis obtained in other studies (Hinck et al. 2009) was not observed. This could mean that mosquitofish might be able to process and eliminate small concentrations of toxins via the liver. Alternatively, the specific chemical compounds that cause physiological and morphological abnormalities in fish could have been absent in the five study streams.

Exposure to WWTP effluents was associated with greater parasitic infection rates in brown trout (*Salmo trutta fario*) {Escher, 1999 #142}. Parasites can also cause behavioral effects such as influencing male mate choice in mosquitofish and reducing shoaling behavior {Deaton, 2009 #144;Tobler, 2008 #141}. Although "black spot disease" (encysted larval trematodes) was not observed in any of the specimens examined in this study, this parasite could cause behavioral abnormalities that would not be readily observable in a field study. Anti-shoaling behavior as well as changes in mate choice can be detrimental to mosquitofish populations, and can increase the probability of predation as well as a decrease in reproductive success.

The pharmaceuticals found during this study have been reported in several other studies of surface waters {Kolpin, 2002 #145;Nunes, 2008 #140;Ramirez, 2009 #99}. Concentrations in the analytes were an order of magnitude less than those found to cause significant abnormalities in lab studies, which could be due to the efficiency of the WWTPs {Ternes, 1998 #149}. Kolpin et al {, 2002 #145} analyzed over 90 organic wastewater contaminants, of which 9 were the same pharmaceuticals found in the Houston bayous, and with median and maximum values of pharmaceuticals higher than values obtained in this study. The one exception is codeine, with concentrations (88 and 100 ηg/L) in Houston that are similar to the median and maximum concentrations (0.012, 0.019 µg/L) reported by Kolpin et al. In future studies, it will be desirable to collect water samples at the same time fish are being collected to confirm actual levels of PPCP exposure. Logistical constraints prevented simultaneous collection of water and fish in the present study.

Sucralose, an artificial sweetener, could be considered an anthropogenic indicator (tracer) compound {Soh, 2011 #146} and was measured at high concentration in Houston bayous. Human tracer compounds are chemicals that are strictly produced and consumed by humans, and it is estimated that a maximum amount of 5 mg/kg/day of sucralose may be consumed in person's lifetime {FDA, 1998 #164}. Sucralose is excreted as the parent compound and can resist degradation through multiple water treatment processes {Roberts, 2000 #147}, which may explain its high concentration in my samples. Caffeine exposure in juvenile fish has caused increased variability of schooling behavior which can increase predation risk {Burgess, 1982 #139}. Other prescription drugs reported in Table 11 have been shown to cause numerous adverse effects, such as alterations of pigmentation in *Gambusia holbrooki* males (Nunes et al. 2008), an increase in the size of the swim bladder in mosquitofish {Nunes, 2008 #140}, and inhibition of antioxidant activities in fish brains {Li, 2011 #148}. As previously stated in the Introduction, these concentrations are generally shown to have biological effects at concentration order of magnitudes higher than those found in the present study.

It should be noted that there are limitations of inference when comparing acute ambient chemical exposure to morphological and physiological indicators. Bayou water samples were collected in 2011, which was later than when the fish were collected (2010). Therefore, the chemical concentrations measured at the sites in 2011 cannot be directly attributed to responses documented in the mosquitofish during the previous summer. It is also important to note that the water samples were collected during the middle of one of the worst droughts in Texas history, which could elevate the concentrations of the compounds {Kolpin, 2004 #152}. Additionally, due to the drought as well as the expense associated with chemical measurement, only one water sample was taken above a WWTP; with this lack of replication, one cannot infer general relationships about the specific sources of chemical pollutants. In the single upstream sample, low levels of chemicals were still found above the WWTP, despite it being the control site. The presence of pharmaceuticals and other household chemicals, such as caffeine, could have been due to leakage from home septic tanks or urban runoff.

4.2 Conclusion

In summary, mosquitofish collected from five streams did not show significant morphological and reproductive abnormalities below WWTP discharges compared to fish above the WWTPs. PPCPs were present in water samples taken from streams below WWTP discharges, however these were at concentrations lower than those shown to impact fish. This study only sampled fish from the uppermost streams in the western suburban area of Houston, and it is expected that results will be different in downstream reaches of the bayous. Nonetheless, this study is the first to document pharmaceutical chemicals and in streams within the bayou system of the Houston metropolitan area, and also the first to evaluate a suite of morphological and reproductive variables in an indicator fish species common in the system.

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